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# Comparison of Detection Methods for *Aspergillus fumigatus* in Environmental Air Samples in an Occupational Environment

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*Methods to study occupational exposures to fungi and fungal materials in facilities where the dominant exposure is the pathogenic and allergenic fungus Aspergillus fumigatus are lacking. Air samples were collected near a conveyor in a wood chip recycling plant to compare methods that might be used to assess exposure to A. fumigatus or suitable proxies. These included total dust mass, total intact spores, culturable propagules growing >35°C, ergosterol, A. fumigatus allergen Asp f1, and quantitative polymerase chain reaction for A. fumigatus. Of these measurements, Asp f1 showed the most promise based on its relative response to measurements where there is a long history of use in industrial hygiene practice (total mass, ergosterol, total intact spores, culturable propagules).*

**Keywords** *Aspergillus fumigatus*, bioaerosols, ergosterol, Asp f1, QPCR, forest products

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## INTRODUCTION

Fungi development on cut wood and wood chips presents an inhalation hazard to workers;<sup>(1)</sup> therefore, accurate, quantitative means for measuring fungal contaminants in workplace atmospheres are essential tools for the industrial hygienist. The wood used in pulp and integrated pulp and paper mills is transported to the mills typically in railcars or trucks. In the late 1950s, a general transition from the shipment of whole logs exclusively to the shipment of wood as chips prepared off site, e.g., at remote chip yards, was begun.<sup>(2,3)</sup> Whether the chips are prepared on or off site, wood chip storage can represent an opportunity for colonization of fungi that may be released to the workplace environment.

Fungi that grow on wood chips are a mixture of thermophilic and thermotolerant taxa and the wood-decay species, including basidiomycetes and soft rot fungi.<sup>(4)</sup> Colonization of the chips

is faster in warmer climates. Since early studies on, the dominant fungus has been *Aspergillus fumigatus*. Concerns about the health effects of molds on wood chips in fuel wood chip furnaces emerged as this technology was adopted in Scandinavia in the 1970s.<sup>(5,6)</sup> A range of diseases has been associated with wood dusts, including many discrete respiratory syndromes. Where hypersensitivity pneumonitis and organic dust toxic syndrome are reported, fungal contaminants of the wood dusts may be a cause, although these have seldom been properly determined in the samples, if any are taken.<sup>(4)</sup>

Environments where wood chips are handled may have airborne values of 10 thousand to 10 million spores/m<sup>3</sup> of air.<sup>(3)</sup> Diseases associated with fungal exposures in the forest products industry include infectious diseases, toxic pneumonitis, airways inflammation, chronic bronchitis, hypersensitivity pneumonitis, asthma, allergic rhinitis, and conjunctivitis.<sup>(3,7,8)</sup> These diseases present an amalgam of symptoms that range from clearly allergic to symptoms related to undefined inflammation.

Although rare, the more serious disease allergic bronchopulmonary aspergillosis (ABPA) may occur in persons who have had a long period of exposure to very high amounts of *A. fumigatus*. The disease is the result of becoming allergic to the fungus, and the risk is increased with the severity of airway enlargement resulting from mucus accumulation (bronchiectasis). In ABPA, *A. fumigatus* grows in mucus, releasing antigens and gliotoxin within the airways resulting in permanent damage to the bronchial wall and surrounding tissue.<sup>(9)</sup> The risk of aspergillosis in the compost worker population is low, although it has been uncommon to employ workers with asthma and other risk factors in these environments.<sup>(10)</sup>

In the case of compost workers who may be at risk of exposure to high concentrations of *A. fumigatus*,<sup>(11)</sup> personal exposures to fungal materials vary according to time, activity, and conditions.<sup>(12)</sup> It is likely that this is also the case in wood chip production and handling facilities; however, methods to

reliably document either a job classification exposure index or personal exposure to *A. fumigatus* within a wood chip (or compost) operation do not exist.<sup>(3)</sup>

The purpose of this study was to compare potential methods for measuring *A. fumigatus* under realistic conditions in a wood chip handling facility so that where warranted, progress on developing fungi exposure information could be collected using measurement methods with characterized limitations.

## MATERIALS AND METHODS

Airborne dust was sampled at a wood chipping facility in Alabama over 2 days in the winter (February 2002). Twenty-one samples were collected at three locations beside a conveyor belt at a hardwood reclamation facility. This site was chosen because it represented an opportunity to collect samples for comparison of analysis methods, not because it represented an area where workers were routinely exposed. Hence, the samples were not intended as measurements of worker exposures. The samples were taken on 102-mm, Teflon-coated, glass fiber filters (#T60A20; Gelman Sciences, Ann Arbor, Mich.) mounted on high-volume air sampling pumps (0.85 m<sup>3</sup>/min; model TF1A Staplex, New York) placed 1.5 m off the ground for about 30 min. Velocity across the sampling plane was measured using a TSI velometer (model 8386; TSI, Saint Paul, Minn.) that was calibrated using a Venturi system (model 400 A, Kurz Instruments, Mosterey, Calif.). The filters were weighed, cut into quarters, and used for analysis of *A. fumigatus* or related materials, specifically (a) the allergen Asp f1; (b) *A. fumigatus* with amplification by quantitative polymerase chain reaction, QPCR; (c) total spores; and (d) ergosterol.

For all measurements, field blanks were analyzed. Additionally, method blanks were performed as required by accrediting bodies or good laboratory practice in the academic laboratories.

Asp f1 was determined using reagents (EL-AF1 Asp f1 ELISA; Biotechnologies, Ltd., Charlottesville, Va.) according to spectrophotometer manufacturer's instructions (PowerWave 200 spectrophotometer; Bio-Tek, Winooski, Va.). The detection limit was 1 ng Asp f1 per sample. Variation between replicates of calibration standards in this method is <5%.<sup>(13)</sup> QPCR analysis for *A. fumigatus* using the primers identified by Haugland<sup>(14)</sup> was performed on the ABI PRISM 7000 Sequence Detection System (ABI, Foster City, Calif.) using standard operating procedure adapted from SOP MERB-020 (National Exposure Research Laboratory, USEPA, Cincinnati, Ohio).

The detection limit was nominally 10 cells/sample. A proportion (5%) of the samples was replicated. Precision in this assay has been reported as  $\pm 4\%$ .<sup>(15)</sup> To determine total intact spores, the filter section was placed in a conical tube containing 8 mL 0.05% (v/v) Tween (Sigma, P1754) in phosphate buffered saline (Cellgro, Herndon, 21-031-CV), vortexed, and counted using a haemocytometer.

The spores in every square were counted; using this method, standard errors of the mean are about 5% and between-slide

variance is virtually nil.<sup>(16)</sup> With vigorous mixing with a wetting agent (Tween) added to the aqueous diluent in the concentration range used, recovery of spores from the filter is associated with <5% variation.<sup>(17)</sup>

Ergosterol was determined after Foto et al.<sup>(18)</sup> using microwave assisted extraction and followed by determination using an Agilent 5973 quadrupole mass spectrometer (Agilent, Santa Clara, Calif.) operating in the electron ionization mode at 70 eV. Compounds were separated using an Agilent 6890 Series gas chromatograph equipped with a 0.25  $\mu\text{m}$  ZB-5 capillary column (5% Phenyl-95% dimethyl-polysiloxane; Phenomenex, Torrance, Calif.). The detection limit in selective ion mode was  $4.5 \pm 0.6$  ng/mL. Analytical variation between replicates of ergosterol-spiked filters was below the ability of the method to detect.

Fifteen additional samples were collected at the field site using an SKC Biosampler (model 225-9597; SKC, Eighty Four, Pa.) at 15 L/min mounted at the same height ca. 0.25 m from the high-volume sampler that collected filter samples used for the other analyses. The pumps were calibrated at the beginning and end of a sample using a calibrated rotameter, and sampling times were recorded. The mean air sample volume was  $0.38 \pm 0.06$  m<sup>3</sup>. The impinger sampler contained 20 mL buffer as above.

After each collection, the impinger container was rinsed with 15 mL buffer and sent to the laboratory under cold conditions arriving within 24 hr. Each sample was serially diluted to extinction in sterile 0.5% Tween over three log dilutions using the spread plate method on 2% malt extract agar plates (Difco). Plates were incubated at 37°C for 2 days, counted, and representative colonies were transferred to 2% malt extract agar and Czapek-Dox agar plates for identification according to the procedures described in the American Industrial Hygiene Association methods manual.<sup>(13)</sup> Variation within these replicates was negligible. Twelve of the samples were collected beside with the filter samples described above.

Data were analyzed using SPSS Version 10. The results from each analytical technique were tested for normality using the Shapiro-Wilks test. All results were log-normally distributed. The log-transformed data were analyzed for Pearson correlations. Values with  $p \leq 0.05$  were considered statistically significant. Additional statistical analyses were done using Systat v. 10.2, mainly to generate the descriptive statistics and verify some of the earlier analyses. Data were excluded only to where there were no complete results within a set.

## RESULTS AND DISCUSSION

Considering the data from culture plates (hereafter "viable"), in all samples, moderately thermotolerant yeasts were typically present in highest concentrations in the 12 samples for which there were side-by-side comparisons with the other measures (mean  $10^7$  CFU/m<sup>3</sup>). Of the filamentous, moderately thermotolerant fungi present, 96% of the colonies of filamentous fungi recovered were *A. fumigatus*.

**TABLE I. Nature of Measurements and Mean Concentrations Observed**

Measures		Mean Concentration (m <sup>-3</sup> ) <sup>A</sup>
QPCR	Cells estimated by DNA	6 × 10 <sup>7</sup>
Asp f1	<i>A. fumigatus</i> allergen	0.9 ng
Total spores	Intact spores	1 × 10 <sup>7</sup> spores
Viable propagules of filamentous fungi at 37°C	Spores, spore/hyphal fragments	1 × 10 <sup>6</sup> colony forming units
Ergosterol	Primary membrane sterol of (most) Ascomycetes	100 ng (~5 × 10 <sup>5</sup> spores) <sup>B</sup>
Mass	Dry weight including debris	5 mg

Notes: The viable propagules were determined from samples collected in an impinger sampler; the remainder of the data were results from filter samplers.

<sup>A</sup> Arithmetic and geometric means and ranges presented in results section.

<sup>B</sup> Estimated using per spore ergosterol values for related aspergillii; Miller and Young.<sup>(16)</sup>

The remaining colonies were almost exclusively *Paecilomyces varioti* (also thermotolerant). Measurement characteristics and overall mean values for each measure are shown in Table I.

Unremarkably, notional cells by QPCR > total intact spores > viable propagules *A. fumigatus* (Hung et al.,<sup>(13)</sup> Miller and Young<sup>(19)</sup>). Because the viable samples were incubated at 37°C, the mesophilic species would not have been recorded. The percentage of viable to total intact spores can fall to as little as 1% for all fungi, but the reduction in spore viability with time for species such as *A. fumigatus* means that a much higher ratio of living vs. dead spores would be present.<sup>(15)</sup> Because the difference is only one order of magnitude less, this indicates that the majority of spores of filamentous fungi present in these air samples were *A. fumigatus*. This may be due to sampling near the wood chip conveyer and that it was winter.

Individual comparisons of samples illuminate a different perspective. Of the measurements taken, the two that provided strictly quantitative exposure data to the fungus were Asp f1 and ergosterol. The remaining methods have more limitations (dust mass) or are not inherently quantitative (viable counts). Asp f1 is the dominant allergen from *A. fumigatus* and is used for medical diagnostic purposes.<sup>(1)</sup> Although there are few measurements, Asp f1 is not normally present in air.<sup>(20)</sup>

In the present study, the arithmetic and geometric means were 0.9 and 0.3 ng/m<sup>3</sup>, respectively (maximum 4 ng/m<sup>3</sup>). The latter value compares with air samples collected over vigorously disturbed leaf samples (range 6.9–29 ng/m<sup>3</sup>).<sup>(21)</sup>

Ergosterol is the primary membrane sterol of ascomycetes and its measurement in environmental samples is a quantitative measure of fungal biomass including in the built

**TABLE II. Correlation of Asp f1 with Other Measures**

	r	n	P
Asp f1			
Intact spores	0.462	20	0.04
Asp f1			
37°C Propagules	0.644	11	0.04
Asp f1			
Ergosterol	0.372	20	0.21
Asp f1			
Mass	0.717	20	<0.001

environment.<sup>(22)</sup> In indoor environments, ergosterol concentrations correlate with areas of visible mold damage.<sup>(18)</sup> In the present study, ergosterol concentrations ranged from 3.4–347 ng/m<sup>3</sup>, with arithmetic and geometric means of 100 g/m<sup>3</sup> and 54 ng/m<sup>3</sup>, respectively. For comparison, in a study of ergosterol of air particulate origin in samples collected from two rooms in 110 homes, ergosterol concentrations ranged from 0.02–11.59 ng/m<sup>3</sup>, with an arithmetic mean of 0.30 ng/m<sup>3</sup>.<sup>(18)</sup> These values are similar to positive values from an earlier study of 400 homes.<sup>(19)</sup>

Correlations of Asp f1 with total recognizable spores, propagules that grew above 37°C, ergosterol, and weight of dust are shown in Table II. Asp f1 positively correlated with dust mass and viable propagules but was not correlated with ergosterol. The ergosterol measurement included a large proportion of ergosterol deriving from yeast; therefore, such a correlation could not be expected.

Correlations among ergosterol with total spores, viable propagules, and dust mass are presented in Table III. Ergosterol was positively correlated with viable propagules and possibly correlated with total spores ( $p = 0.085$ ). The latter result is consistent with the lack of correlation of Asp f1 with ergosterol because, again, ergosterol is also present in yeast cells.

However, the observation that ergosterol-total spore correlation almost reached statistical significance indicates that the majority of the ergosterol in the dust was derived from biomass from filamentous fungi, not yeasts. Ergosterol was correlated with the total viable propagules that grew above 37°C (Table III), and which consisted almost entirely of *A. fumigatus* (96%). This in turn is supported by the observation that Asp f1 was weakly correlated with total spores (Table II).

**TABLE III. Correlation of Ergosterol with Other Measures**

	r	n	p
Ergosterol			
Intact spores	0.612	18	0.085
Ergosterol			
37°C propagules	0.723	11	0.01
Ergosterol			
Mass	0.501	20	0.05

**TABLE IV. Correlation of QPCR with Other Measures**

	r	n	p
QPCR			
Spores	0.426	18	0.16
QPCR			
37°C Propagules	0.330	11	0.32
QPCR			
Ergosterol	0.004	18	>.99
QPCR			
Mass	0.258	18	0.31

Ergosterol was weakly correlated with dust mass indicating that a large proportion of the particulate matter above the conveyers was fungal in origin, but also present (as would be expected) were debris, presumably wood dusts, and soil.

## CONCLUSIONS

In summary, the quantitative data on Asp f1, ergosterol, and dust mass are within a reasonable range consistent with existing literature, and the correlations between them make biological sense and are internally consistent. This implies a validity/reliability in the sampling, the division of samples for analysis, and the analyses all done by independent laboratories. The data from which these correlations were drawn from 11 to 20 samples, sufficient to produce reliable conclusions.

There were no statistically significant correlations between QPCR and the other measurements (Table IV). For some of the reasons listed above, it is not surprising that there were no correlations with ergosterol or mass; namely, that the ergosterol measurement included yeast-derived ergosterol, and the mass included debris and soil. The failure of QPCR values to correlate with intact spores is more surprising, since the latter measure correlated with both Asp f1 and viable propagules. Over 96% of this analytical result comprised viable propagules of *A. fumigatus*, each one by definition contained *A. fumigatus* DNA.

There are few specific data on relationships between viable cells and the QPCR measurement by the technique used in the present study beyond the generalization that the QPCR values are higher. In a study from Meklin et al.,<sup>(23)</sup> their Figure 1 shows large differences in ratio between QPCR and propagules of *A. fumigatus* in individual experiments. Their lack of correlation between *A. fumigatus* propagules and the QPCR data is similar to ours, which may relate to the presence of QPCR inhibitors inherent to particular strains of this fungus<sup>(13,24,25)</sup> and/or the presence of contaminants from the air sample in a given environment in the filter DNA extracts.<sup>(13,25)</sup> As noted above, the air filters from this industrial environment collected material amounts of wood dust, soil, and probably engine-derived particulate matter.

To our knowledge, this is the first study to compare a variety of measurements for assessing fungal exposure in

the strict sense in an occupational environment. We found that Asp f1 correlated with viable propagule counts of *A. fumigatus*. Because both the allergen and viable propagules are important in considering the disease outcome of this fungus, this measurement deserves more study. The sensitivity of Asp f1 determinations may be improved with better extraction techniques or other means. Where *A. fumigatus* is the dominant fungus, such as in the context studied, ergosterol may also be a suitable marker of exposure. In this study, QPCR did not provide a reliable measure of exposure for industrial hygiene studies in this occupational environment.

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